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14. ABSTRACT Our research program is to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject is to understand the function of a novel estrogen receptor variant, ER- α 36, in antiestrogen resistance. In the past year, we have made significant progress towards accomplishment of the tasks proposed. We demonstrated that antiestrogen resistant ER-positive breast cancer cells contain high populations of stem/progenitor cells, and the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. We also found that ER- α 36 plays an important role in positive regulation of both ER-positive and -negative breast cancer stem/progenitor cells and contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens. Further study of the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance will not only provide important information about the function of breast cancer stem/progenitor cells in development of antiestrogen resistance, but will also lay the foundation for development of novel therapeutic approaches to interfere with antiestrogen resistance.					
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INTRODUCTION

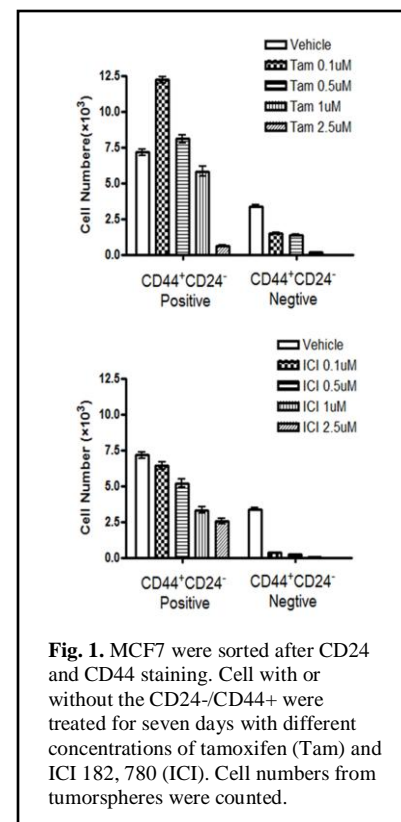
Our research program is to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance of a novel estrogen receptor variant, ER- α 36, in resistance of breast cancer stem/progenitor cells to antiestrogens. In the past year, we have made significant progress towards accomplishment of the works proposed in the original grant. We demonstrated that antiestrogen resistant ER-positive breast cancer cells contain high populations of stem/progenitor cells, and the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens. The effects of antiestrogens on the ER-positive breast cancer stem/progenitor involve changes of both proliferation and differentiation. We also found that ER- α 36 plays an important role in positive regulation of both ER-positive and -negative breast cancer stem/progenitor cells and contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens. Further study of the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance will not only provide important information about the function of breast cancer stem/progenitor cells in development of antiestrogen resistance, but will also lay the foundation for development of novel therapeutic approaches to interfere with antiestrogen resistance.

BODY

Task 1: To determine whether the breast cancer stem/progenitor cells from ER-positive breast cancer cells are involved in antiestrogen resistance and the function of ER- α 36 in the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens (months 1-16).

1a. *To examine the effects of antiestrogens on the stem/progenitor cells enriched from the ER-positive breast cancer cells, MCF7, T47D and ZR-75-1 using the colony formation assay (months 1-4, from SOW).*

We have used ALDEFLUOR sorting and CD24-/CD44+ staining to enrich breast cancer stem/progenitor cells from ER-positive breast cancer cells, MCF7, T47D and ZR-75-1, and tested the effects of antiestrogens on these cells. Originally, we performed the colony formation assay as proposed in the grant, but found that attachment of stem/progenitor cells to culture dish resulted in loss of the stemness and gain of differentiation. To solve this problem, we changed to stem cell culture medium and low-attachment dishes. After antiestrogen treatment, the cells grown on low-attachment dishes were dissociated and the cell number was counted. We found that the breast cancer stem/progenitor cells enriched using the sorting method with ALDH1 or CD24-/CD44+ as makers showed less sensitivity to antiestrogens TAM and Fulvestrant (ICI182, 780) compared to cells without these markers (**Fig. 1 and data not shown**). In MCF7 cells, TAM at 0.1 μ M even stimulated proliferation of the CD24-/CD44+ positive cells (**Fig. 1**). Our results thus suggested that ER-positive breast cancer stem/progenitor cells with the ALDH1 and CD24-/C44+ markers are insensitive to antiestrogens.



1b. To assess the populations of the stem/progenitor cells in HER2 expressing breast cancer cells MCF7/TAM, BT474 and MCF7/HER-2/18 using the ALDEFLUOR kit and flow-cytometry, and compare to antiestrogen sensitive cells such as MCF7 (months 1-4, from SOW).

To examine the populations of the stem/progenitor cells in HER2-expressing breast cancer cells MCF7/TAM, BT474 and MCF7/HER-2/18-2/18, we used the ALDEFLUOR assay and the tumorsphere formation assay. We used MCF7 cells as a control. We found that ALDH1-high population was increased in these HER2 expressing cells (**data not shown**). The tumorspheres formed by these cells were dissociated and the cell number was counted. We found that MCF7/TAM, BT474 and MCF/HER-2/18 cells contain significantly higher populations of stem/progenitor cell compared to MCF7 cells (**Fig. 2**). Our results thus indicated that the antiestrogen insensitive and HER-expressing cells contain high population of breast cancer stem/progenitor cells.

1c. To examine the effects of antiestrogens on the abilities of ER-positive breast cancer cells to form tumorspheres and self-renewal (months 3-8, from SOW).

ER-positive breast cancer cells were used for tumorsphere formation assays. The tumorsphere cultures were performed in stem cell medium. Cells were plated at low density (10,000 viable cells/well) in 6-well ultra-low attachment plates for seven days in the absence and presence of different concentrations of TAM and fulvestrant. The numbers of tumorspheres formed were dissociated into single cells and the cell number was counted. We found that cells from tumorspheres were more resistant to both antiestrogens compared to the parental cells grown on regular dishes (**Fig. 3**). Interestingly, In the presence of both TAM and ICI 182,780 at low concentrations (0.1-1 μM), there were increased numbers of stem/progenitor cells from MCF7 and T47D cells, suggesting that antiestrogens may increase the populations of stem/progenitor cells from ER-positive breast cancer cells. The stem/progenitor cells enriched from MCF7/TAM cells were more potently stimulated by TAM at concentrations from 0.1-0.5 μM compared to MCF7 cells (**Fig. 3**). The primary tumorspheres were dissociated and passaged to form secondary and third generation of tumorspheres in the presence and absence of both antiestrogens to test the effects of antiestrogens on the ability of self-renewal of these cells. We found that the control vehicle did not significantly influence long-term

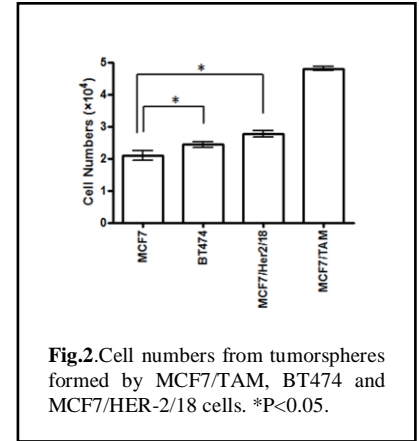


Fig.2. Cell numbers from tumorspheres formed by MCF7/TAM, BT474 and MCF7/HER-2/18 cells. *P<0.05.

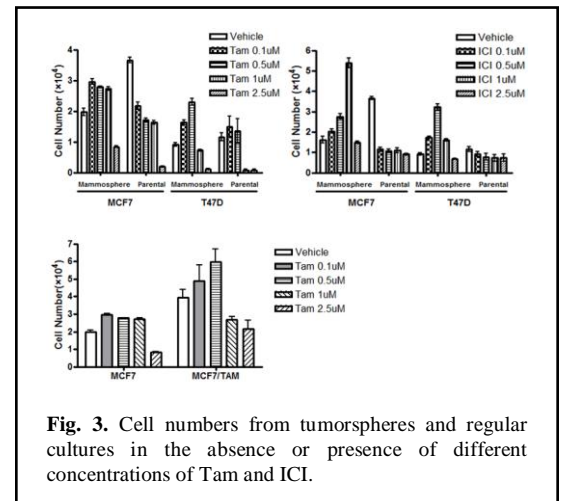


Fig. 3. Cell numbers from tumorspheres and regular cultures in the absence or presence of different concentrations of Tam and ICI.

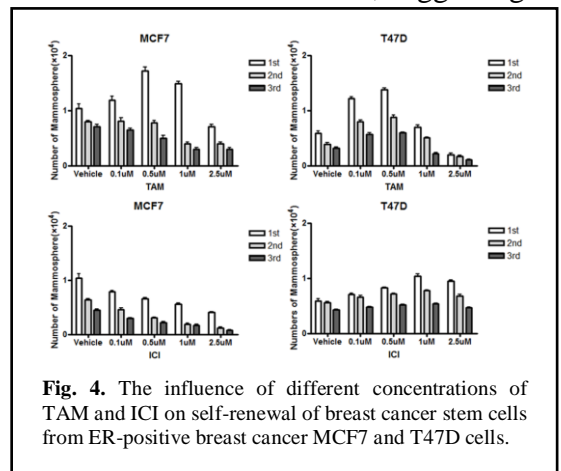


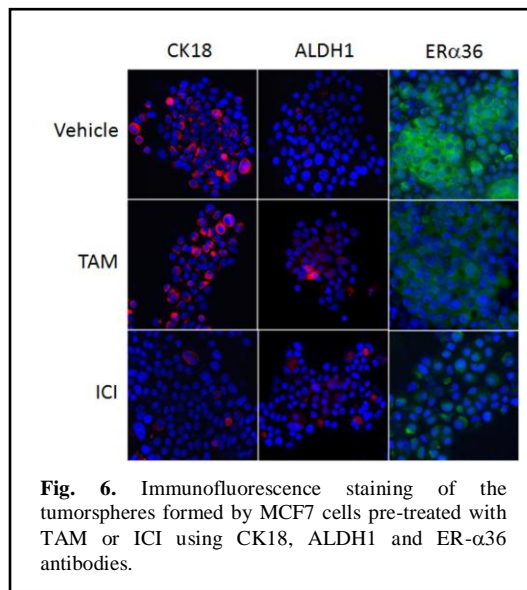
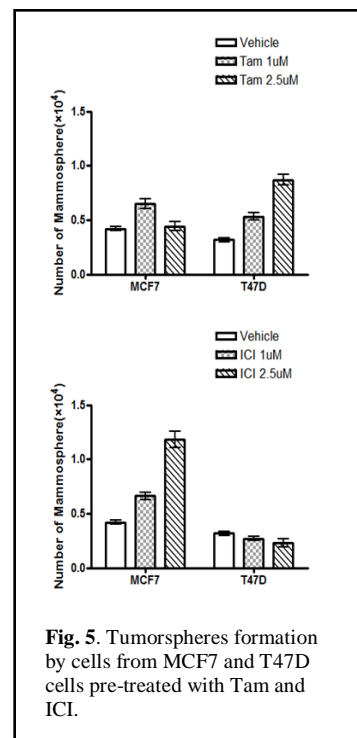
Fig. 4. The influence of different concentrations of TAM and ICI on self-renewal of breast cancer stem cells from ER-positive breast cancer MCF7 and T47D cells.

self-renewal of these cells. However, in second and third generations of tumorspheres, T47D cells retained stimulatory response to low concentrations of both antiestrogens while MCF7 cells showed a decrease in self-renewal capabilities of the stem/progenitor cells in the presence of both antiestrogens (**Fig. 4**). Our results indicated that antiestrogens were able to stimulate the populations of ER-positive stem/progenitor cells and to influence the self-renewal capabilities of breast cancer stem/progenitor cells.

1d. To examine whether antiestrogen treatment selectively enriches the stem/progenitor cells from ER-positive breast cancer cells using the tumorsphere formation assay and immunofluorescence staining (months 3-8, from SOW).

To examine whether antiestrogen treatment selectively enriches the stem/progenitor cells from ER-positive breast cancer cells, we treated MCF7 and T47 cells in regular culture dishes with 1 μ M of TAM and ICI 182, 780 for three days. The cells survived antiestrogen treatment were then transferred to stem cell culture medium and low-attachment dishes and cultured for seven days. The numbers of tumorspheres formed were then counted. After TAM or ICI 182, 780 treatment, the survived cells from MCF7 cells generated more tumorspheres than MCF7 cells treated with control vehicle. T47D cells, however, were more sensitive to ICI 182, 780 (ICI) than to TAM (**Fig. 5**). Thus, our data indicated that ER-positive breast cancer cells survived antiestrogen treatment contain high population of stem/progenitor cells, and also suggested that ER-positive breast cancer cells may contain sub-populations of stem/progenitor cells that respond to different antiestrogens differently.

We also performed immunofluorescence staining of the tumorspheres formed by MCF7 and T47D cells pretreated with TAM or ICI using CK18, ALDH1 and ER- α 36 antibodies. We found that ER- α 36 is highly expressed in tumorspheres formed by MCF7 and T47D cells treated with vehicle but not with TAM and ICI (**Fig. 6** and **data not shown**). In MCF7 cells, treatment of both TAM and ICI at 1 μ M significantly increased the number of cells positive for ALDH1 (**Fig. 6**), consistent with the finding that both antiestrogens increased the number of tumorspheres. In T47D cells, we found that TAM treatment increased ALDH1 positive cells but ICI failed to influence the number of the ALDH1 positive cells (**data not shown**). Interestingly, in both cell lines, TAM treatment increased the number of CK18 positive cells while ICI decreased CK18 positive cells (**Fig. 6**, and **data not shown**), suggesting TAM treatment stimulates proliferation of ER-positive breast cancer stem/progenitor cells that produce more number of differentiated cells and ICI inhibits differentiation of breast cancer stem/progenitor cells.



1e. To establish stable cell lines from MCF/TAM and MCF7/HER-2/18 cells with ER- α 36 expression “knocked-down” using the shRNA approach (months 2-6, from SOW).

We have successfully established a stable cell line (MCF7/TAM/Si36) from MCF7/TAM cells with the expression levels of ER- α 36 knocked-down by ER- α 36 shRNA. We found TAM resistant MCF7 cells became TAM sensitive when the expression levels of ER- α 36 were knocked-down (see **Appendix**), indicating that elevated level of ER- α 36 expression is one of the underlying mechanisms of TAM resistance. We, however, failed to establish stable cell lines from MCF7/HER-2/18 cells with several attempts due to the fact that this cell line carrying a HER2 expression vector that shares the same selection marker with the ER- α 36 shRNA expression vector. Recently, our laboratory established a Lentivirus-based ER- α 36 shRNA system. We will use this system to express ER- α 36 shRNA in MCF7/HER-2/18 cells. We expect we will successfully establish stable cell lines from MCF7/HER-2/18.

1f. To investigate and compare the differentiation lineages of ER-positive breast cancer stem/progenitor cells resistant to tamoxifen and fulvestrant (months 6-10, from SOW).

To investigate and compare the differentiation lineages of ER-positive breast cancer stem/progenitor cells pretreated with antiestrogens, tumorspheres formed by ER-positive breast cancer MCF7 and T47D cells pre-treated with antiestrogens at 1 μ M were disrupted and single cell suspensions were plated on collagen-coated coverslips and maintained in serum containing medium for five days, and indirect immunofluorescence assay was performed to determine differentiation lineages of these cells using cytokeratin 18 for epithelial cells, CD10 for myoepithelial cells. We found that TAM pre-treatment increased the number of cells expressing CK18 and CD10 while ICI 182, 780 pre-treatment decreased the number of differentiation cells (**Fig. 7**), and ER-positive breast cancer T47D cells showed a similar result (**Fig. 7**), suggesting that TAM may induce differentiation of ER-positive breast cancer stem/progenitor cells into both luminal and myoepithelial cell lineages. ICI 182, 780, on the contrary, inhibited differentiation, indicating that TAM and ICI 182, 780 have different effect on differentiation of the breast cancer stem/progenitor cells.

Interestingly, when TAM resistant MCF7 cells (MCF7/TAM) were used to examine the effects of antiestrogens on differentiation of the breast cancer stem/progenitor cells, we found that TAM inhibited the differentiation of the breast cancer stem/progenitor cells enriched from MCF7/TAM cells (**Fig. 8**), suggesting escape of TAM-induced

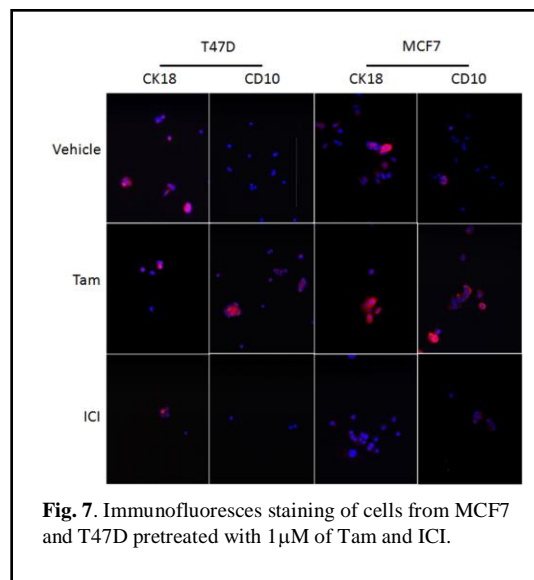


Fig. 7. Immunofluorescence staining of cells from MCF7 and T47D pretreated with 1 μ M of Tam and ICI.

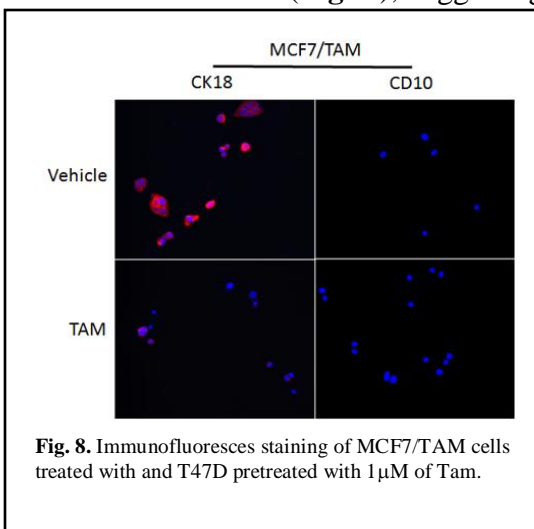


Fig. 8. Immunofluorescence staining of MCF7/TAM cells treated with and T47D pretreated with 1 μ M of Tam.

differentiation of breast cancer stem/progenitor cells is one of the mechanisms underlying TAM resistance.

1g. To study the abilities of the stem/progenitor cells enriched from HER2 expressing cells to form tumorspheres, self-renewal and differentiation (months 10-14, from SOW).

We examined the abilities of the stem/progenitor cells enriched from HER2 expressing cells to form tumorspheres and self-renewal. BT474 and MCF7/Her2/18 cells were used for tumorsphere formation assays. The tumorsphere cultures were performed in stem cell medium. Cells were plated at low density (10,000 viable cells/well) in 6-well ultra-low attachment plates for seven days in the absence and presence of different concentrations of TAM and fulvestrant. The numbers of tumorspheres formed were counted using a Multisizer 3 Coulter Counter (Beckman Coulter, size > 40 μ M). We found that the numbers of tumorspheres formed by both cell lines were decreased with increased concentrations of TAM and ICI (Fig. 9). The primary tumorspheres were dissociated and passaged to form secondary and third generation of tumorspheres in the presence and absence of different antiestrogens to test the effects of antiestrogens on the ability of self-renewal of these cells. We found that in second and third generations of tumorspheres, both cells showed a decrease in self-renewal capabilities of the stem/progenitor cells in the presence of increased concentrations of both antiestrogens (Fig. 9). Our results indicated that antiestrogens were able to negatively influence the self-renewal capabilities of these HER-2 expressing breast cancer stem/progenitor cells.

The effects of different concentrations of TAM and ICI 182,780 on differentiation of the stem/progenitor cells enriched from HER-2 expressing BT474 and MCF7/Her2/18 cells are currently being studied.

1h. To perform in vivo assays to assess tumor seeding efficiency of the antiestrogen resistant stem/progenitor cells from ER-positive breast cancer cells; 240 female nude mice will be used (months 10-16, from SOW).

We have performed a pilot experiment to provide proof of concept. To assess tumor seeding efficiency of the MCF7 cells survived 1 μ M TAM, presumably the stem/progenitor cells. TAM resistant MCF7 cells at different dilutions of 1×10^2 , 1×10^3 , 1×10^4 and 1×10^5 were re-suspended in 0.1 ml of Matrigel and inoculated subcutaneously into the mammary fatpad of ovariectomized female nude mice (5-6 weeks old, strain CDI nu/nu, Charles River Breeding Laboratory). The mice were also implanted with 0.35 mg/60-day slow-release pellets (Innovative Research) to facilitate tumor formation of these cells. A group of four mice were used. MCF7 survived 1 μ M TAM treatment exhibited potent tumor seeding efficiency; generating tumors at 100 cells

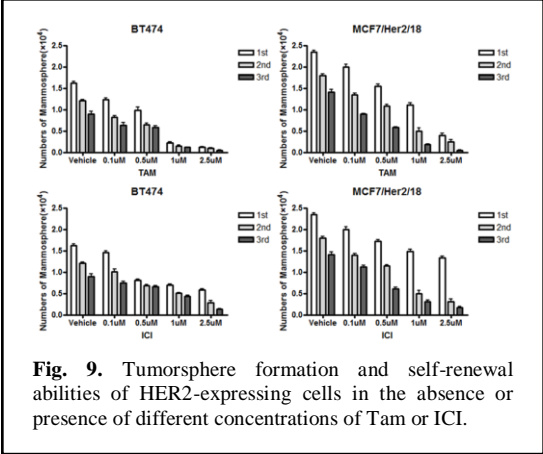


Fig. 9. Tumorsphere formation and self-renewal abilities of HER2-expressing cells in the absence or presence of different concentrations of Tam or ICI.

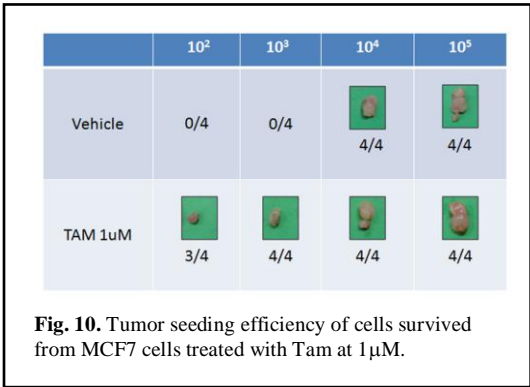


Fig. 10. Tumor seeding efficiency of cells survived from MCF7 cells treated with Tam at 1 μ M.

while MCF7 cells treated with vehicle required 10,000 cells to generate tumors (**Fig. 10**). Our results thus strongly suggested that TAM pretreatment enriched tumor-initiating cells, i.e. breast cancer stem/progenitor cells, which provided the proof of concept for our future experiments.

Due to a large-scale experiment was conducted in the past year that limited the space of our animal facility; we were unable to perform all of the experiments at one time. With the accomplishment of that project now, we have purchased mice and are ready to perform the experiments proposed. We do not expect a delay for this experiment.

li. To examine ER- α 36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens using cell lines with forced expression and down-regulated expression of ER- α 36 (months 8-14, from SOW).

We first examined the function and underlying mechanism of ER- α 36 in TAM resistance. We found that tamoxifen induced ER- α 36 expression and tamoxifen resistant MCF7 cells expressed high levels of ER- α 36. In addition, MCF7 cells with forced expression levels of ER- α 36 and H3396 cells expressing high levels of endogenous ER- α 36 were insensitive to tamoxifen. Knockdown of ER- α 36 expression in tamoxifen resistant cells with the shRNA method restored tamoxifen sensitivity. We also found tamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER- α 36 expressing cells. Finally, we found that cells with high levels of ER- α 36 expression were hypersensitive to estrogen; activating ERK phosphorylation at pM range (see **Appendix**). Our results strongly indicated that ER- α 36 plays an important role in development of antiestrogen resistance. Elevated ER- α 36 expression is one of the mechanisms underlying the development of TAM resistance presumably through mediating agonist activity of TAM.

To determine if alterations of ER- α 36 expression affects stem cell population, the cells with different levels of ER- α 36 expression were examined for populations of the stem/progenitor cells using flowcytometry analysis after the ALDEFLUOR or CD44/CD24 staining. We found that cells with high levels of ER- α 36 contain higher populations of stem/progenitor cells while cells with knocked-down levels of ER- α 36 have a decreased population of stem/progenitor cells (**data not shown**). When tumorsphere formation assays were performed in these cells, we found that MCF7 cells with forced ER- α 36 expression produced more number and bigger sized tumorspheres compared to the MCF7 control cells. MCF7 cells with knocked-down levels of ER- α 36 expression had significant less tumorspheres compared to the MCF7 control cells (**Fig. 11**). When these stem/progenitor cells were challenged with antiestrogens, we found that stem/progenitor cells with high levels of ER- α 36 were more resistant to both TAM and ICI 182, 780 (**Fig. 11**). Our results suggested that ER- α 36 is involved in positive regulation of breast cancer stem/progenitor cells and high levels of ER- α 36 expression contributes to the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens.

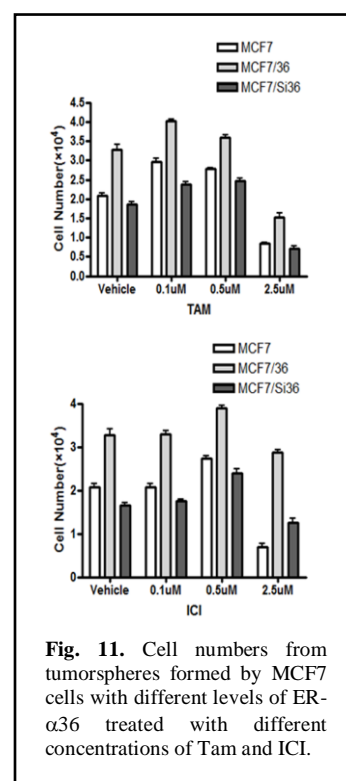


Fig. 11. Cell numbers from tumorspheres formed by MCF7 cells with different levels of ER- α 36 treated with different concentrations of Tam and ICI.

The experiments to examine the differentiation and self-renewal of the breast cancer stem/progenitor cells enriched from MCF7 cells with different levels of ER- α 36 are currently underway in our laboratory.

Task 2: To investigate the function and the underlying mechanisms of ER- α 36 in antiestrogen resistance of the ER-negative breast cancer stem/progenitor cells (months 13-24).

We have also made some progress in the projects proposed for the task 2, which provided the proof of concept and the framework for the successful accomplishment of this task in the next funding year.

2a. To determine whether ER-negative breast cancer cells have increased populations of stem/progenitor cells compared to their variants with the knocked-down levels of ER- α 36 expression (months 13-14, from SOW).

To determine the role of ER- α 36 in maintenance of the stem/progenitor cells from ER-negative breast cancer cells, we used ER-negative breast cancer MDA-MB-231 and SK-BR-3 cells as well as these cells with knocked-down levels of ER- α 36 expression. These cells were examined for populations of the stem/progenitor cells using flowcytometry analysis after the ALDEFLUOR staining. We found that cells with knocked-down levels of ER- α 36 contain a decreased population of ALDH1-positive cells (**Fig. 12**; Kang *et al.*, 2011). Recently, we also reported knockdown of ER- α 36 expression in ER-negative breast cancer MDA-MB-231 cells severely impaired the ability of these cells to form tumor *in vivo* (Zhang *et al.*, 2011). Taken together, our results demonstrated that ER- α 36 is important in maintenance of the stem/progenitor cells from ER-negative breast cancer cells and contributes to the tumor-initiating capability of ER-negative breast cancer.

We are currently investigating the role of ER- α 36 in the self-renewal and differentiation of ER-negative breast cancer stem/progenitor cells as proposed in the grant.

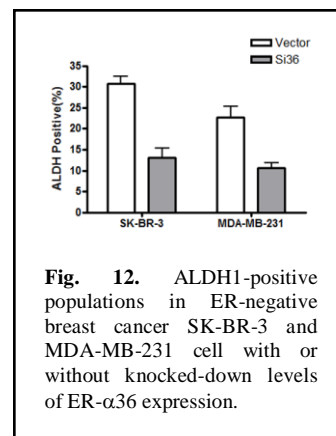


Fig. 12. ALDH1-positive populations in ER-negative breast cancer SK-BR-3 and MDA-MB-231 cell with or without knocked-down levels of ER- α 36 expression.

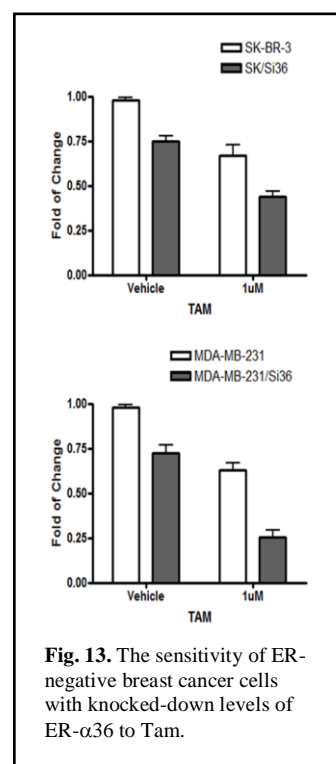


Fig. 13. The sensitivity of ER-negative breast cancer cells with knocked-down levels of ER- α 36 to Tam.

2b. To examine the effects of antiestrogens on the enriched stem/progenitor cells from the ER-negative breast cancer cells with different levels of ER- α 36 expression and compare to un-enriched ER-negative breast cancer cells to determine if ER-negative stem/progenitor cells are more resistant to antiestrogens (months 13-16, from SOW).

To examine the effects of antiestrogens on the stem/progenitor cells enriched from the ER-negative breast cancer cells with knocked-down levels of ER- α 36 expression, we employed tumorsphere formation assays using SK-BR-3 and MDA-MB-231 cells transfected with ER- α 36 shRNA expression vector. Cells were plated at low density (5,000 viable cells/well) in 6-well ultra-low attachment plates for seven days in the absence and presence of different concentrations of TAM. The tumorspheres formed were dissociated into single cells and the cell number was counted. We found that ER-negative breast cancer cells with knocked-down levels

of ER- α 36 were more sensitive to TAM (**Fig. 13**). This data thus suggested that downregulation of ER- α 36 sensitizes ER-negative breast cancer cells to antiestrogens.

Currently, we are investigating the effects of antiestrogens on the self-renewal and differentiation of the stem/progenitor cells from ER-negative breast cancer cells with knocked-down levels of ER- α 36 expression.

2c. To determine if ER- α 36 can be used as a novel marker for breast cancer stem/progenitor cells (18-24, from SOW).

We examined the possibility to use ER- α 36 as a surface marker for breast cancer stem/progenitor cells to sort ER- α 36 highly expressing cells and to examine the stemness of these cells. Cells were stained with our polyclonal ER- α 36 antibody labeled with the Alexa Fluor dye and sorted with flowcytometry. When these ER- α 36-high cells were examined for the stem/progenitor cell characteristics, we found this ER- α 36 specific antibody inhibited growth of sorted cells, consistent with our recent report the ER- α 36 specific antibody inhibited the growth of ALDH1-positive cells from ER-negative breast cancer SK-BR-3 cells (Kang *et al.*, 2011). Our data suggested that the ER- α 36 specific antibody might be developed into a novel therapeutic agent by targeting breast cancer stem/progenitor cells.

We are currently screening a library of ER- α 36 specific monoclonal antibodies to identify antibodies that are able to specifically recognize ER- α 36 but unable to inhibit cell growth.

2d. To investigate the underlying mechanisms of ER- α 36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens (months 17-24, from SOW).

Our recent studies have provided a framework to investigate the underlying mechanisms of ER- α 36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens. We recently reported that ER- α 36 mediates agonist activities of both TAM and ICI182, 780 in ER-negative breast cancer cells (Zhang *et al.*, 2012) and ER- α 36 mediates TAM-induced AKT phosphorylation in TAM resistant MCF7 cells (**see Appendix**). These results strongly suggested that ER- α 36-mediated PI3K/AKT signaling induced by antiestrogens is one of the mechanisms by which the breast cancer stem/progenitor cells that express high levels of ER- α 36 become antiestrogen resistant. In the next year of funding, we will focus our research on the PI3K/AKT signaling pathway to examine the possibility of down-regulation of ER- α 36 expression or inhibition of the PI3K/AKT pathway will sensitize antiestrogen resistant breast cancer stem/progenitor cells to antiestrogens.

KEY RESEARCH ACCOMPLISHMENTS

1. We demonstrated that antiestrogen-resistant and HER-2 expressing ER-positive breast cancer cells contain high populations of stem/progenitor cells.
2. We found that the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells are refractory to and even stimulated by antiestrogens.
3. We found that antiestrogens influence both proliferation and differentiation of the ER-positive breast cancer stem/progenitor cells.
4. We also found that a novel estrogen receptor variant, ER- α 36, plays an important role in positive regulation of both ER-positive and -negative breast cancer stem/progenitor cells and contributes to the resistance of breast cancer stem/progenitor cells to antiestrogens presumably through mediating agonist activities of antiestrogens.

5. We discovered that the anti-ER- α 36 specific antibody inhibits proliferation of both ER-negative and -positive breast cancer stem/progenitor cells.

REPORTABLE OUTCOMES

Zhang, XinTian and Wang, Z-Y “Estrogen Receptor- α Variant, ER- α 36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity”. *Submitted*.

CONCLUSIONS

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen (TAM) and fulvestrant (ICI 182, 780, ICI) provides a successful treatment option for ER-positive breast cancer patients in the past two decades. However, despite the significant anti-neoplastic activity of antiestrogens, most breast tumors are eventually resistant to antiestrogen therapy, which largely affects the efficacy of antiestrogen treatment. The exact mechanisms underlying the antiestrogen resistance in these ER-positive tumors have been long waited. Several mechanisms have been postulated to be involved in the TAM resistance such as increased growth factor signaling, metabolism of TAM by CYP2D6 variants, altered expression of co-regulators, mutations of ER- α .

In this grant, we proposed to study the role and underlying mechanisms of breast cancer stem/progenitor cells in antiestrogen resistance. One central subject of this study is to understand the biological significance of a novel estrogen receptor variant, ER- α 36, in resistance of breast cancer stem/progenitor cells to antiestrogens. In the past year, we have made significant progress towards accomplishment of the works proposed in the original grant.

We discovered that antiestrogen resistant ER-positive breast cancer cells such as ER-positive breast cancer MCF7 cells selected through long-term culture of cells in the presence of TAM, and cells expressing high levels of growth factor receptor HER-2 such as BT474 and MCF7/HER-2/18 contain high populations of stem/progenitor cells. These results provided evidence to support the hypothesis that breast cancer stem/progenitor cells are involved in antiestrogen resistance, and increased populations of breast cancer stem/progenitor cells is a novel and important mechanism underlying development of antiestrogen resistance.

We further demonstrated that the stem/progenitor cells enriched from antiestrogen sensitive ER-positive breast cancer cells were refractory to antiestrogens TAM and ICI. Antiestrogens TAM and ICI at low concentrations ($< 1\mu\text{M}$) even stimulated proliferation of the stem/progenitor cells. Immunofluorescence staining showed that both TAM and ICI treatment increased the number of ALDH1 cells, suggesting an increase of the stem/progenitor cells. TAM treatment also increased the number of CK18 positive cells while ICI decreased the number of CK18 positive cells, suggesting that TAM may increase the lineage-specific progenitor cells and ICI may inhibit differentiation of breast cancer stem cells. To our knowledge, this is the first time to show that breast cancer stem/progenitor cells are resistant to antiestrogens and antiestrogens influence both proliferation and differentiation of breast cancer stem/progenitor cells. Thus, it is possible that antiestrogen treatment specifically selects and enriches breast cancer stem/progenitor cells that are resistant to antiestrogens. This suggests that antiestrogen therapy, while killing the bulk of breast tumor cells, may eventually fail since they do not eradicate breast cancer stem cells that survive to regenerate new tumors.

We also found that a novel estrogen receptor variant, ER- α 36, plays an important role in positive regulation of both ER-positive and -negative breast cancer stem/progenitor cells and is involved in the resistance of breast cancer stem/progenitor cells to antiestrogens, presumably

through mediating agonist activities of antiestrogens. These findings are of both biological and clinical significance.

Many signaling pathways involved in regulation of normal stem cell fate, self-renewal, and maintenance including Hedgehog, Bmi-1, Wnt, NOTCH, HER-2, p53 and PTEN/Akt/ β -catenin signaling have been identified to play roles in breast cancer stem/progenitor cell (Korkaya *et al.*, 2008 & 2009). However, the involvement of estrogen signaling, a major signaling pathway in breast cancer development, in regulation of breast cancer stem/progenitor cells has not been established, mainly because expression of estrogen receptor- α (ER- α) in breast cancer stem/progenitor cells remains controversial. It was reported that stem cells isolated from normal mammary gland and breast cancer tissues lack expression of the full-length ER- α (Sleeman *et al.*, 2006). Our results for the first time demonstrated that breast cancer stem/progenitor cells expressed ER- α 36, a variant of ER- α and the non-genomic estrogen signaling mediated by ER- α 36 plays an important role in positive regulation of breast cancer stem/progenitor cells. Further research will provide novel and important information about the function and underlying mechanism of non-genomic estrogen signaling in breast cancer stem/progenitor cells from both ER-positive and -negative breast cancer.

The discovery of breast tumor cells that behave like stem cells and that are resistant to chemotherapy drugs, radiation therapy and antiestrogens provided a reasonable explanation for the difficulty to eradicate breast cancer. Novel mechanisms and targets for development of effective therapeutic approaches to inhibit growth of breast cancer stem cells are urgently needed. Our finding that the ER- α 36 specific antibody blocked growth of breast cancer stem/progenitor cells provided a rational to development of novel therapeutic approaches by targeting ER- α 36, which will ultimately revolutionize current therapeutic approaches.

During the next year of funding, it is planned to finish the task 1 and further pursue the work of task 2 in my Approved Statement of Work.

Task 1: To determine whether the breast cancer stem/progenitor cells from ER-positive breast cancer cells are involved in antiestrogen resistance and the function of ER- α 36 in the resistance of ER-positive breast cancer stem/progenitor cells to antiestrogens (months 1-16).

1e. To establish stable cell lines from MCF/TAM and MCF7/HER-2/18 cells with ER- α 36 expression “knocked-down” using the shRNA approach (months 2-6, from SOW).

We will use the Lentivirus-based ER- α 36 shRNA system to express ER- α 36 shRNA in MCF7/HER-2/18 cells and establish stable cell lines from MCF7/HER-2/18.

1g. To study the abilities of the stem/progenitor cells enriched from HER2 expressing cells to form tumorspheres, self-renewal and differentiation (months 10-14, from SOW).

We will finish the examination of the effects of different concentrations of TAM and ICI 182,780 on differentiation of the stem/progenitor cells enriched from HER-2 expressing BT474 and MCF7/Her2/18 cells.

1h. To perform in vivo assays to assess tumor seeding efficiency of the antiestrogen resistant stem/progenitor cells from ER-positive breast cancer cells; 240 female nude mice will be used (months 10-16, from SOW).

We will finish all of the *in vivo* assays to assess tumor seeding efficiency of the antiestrogen resistant stem/progenitor cells from ER-positive breast cancer cells.

1i. To examine ER- α 36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens using cell lines with forced expression and down-regulated expression of ER- α 36 (months 8-14, from SOW).

We will finish the examination of the differentiation and self-renewal of the breast cancer stem/progenitor cells enriched from MCF7 cells with different levels of ER- α 36.

Task 2: To investigate the function and the underlying mechanisms of ER- α 36 in antiestrogen resistance of the ER-negative breast cancer stem/progenitor cells (months 13-24).

2a. To determine whether ER-negative breast cancer cells have increased populations of stem/progenitor cells compared to their variants with the knocked-down levels of ER- α 36 expression (months 13-14, from SOW).

We will study the role of ER- α 36 in the self-renewal and differentiation of ER-negative breast cancer stem/progenitor cells as proposed in the grant.

2b. To examine the effects of antiestrogens on the enriched stem/progenitor cells from the ER-negative breast cancer cells with different levels of ER- α 36 expression and compare to un-enriched ER-negative breast cancer cells to determine if ER-negative stem/progenitor cells are more resistant to antiestrogens (months 13-16, from SOW).

We will investigate the effects of antiestrogens on the self-renewal and differentiation of the stem/progenitor cells from ER-negative breast cancer cells with or without knocked-down levels of ER- α 36 expression.

2c. To determine if ER- α 36 can be used as a novel marker for breast cancer stem/progenitor cells (18-24, from SOW).

We are currently screening a library of ER- α 36 specific monoclonal antibodies to identify antibodies that are able to specifically recognize ER- α 36 but unable to inhibit cell growth. We will then use this antibody to enrich ER- α 36-high cells and to test their stem cell characteristics.

2d. To investigate the underlying mechanisms of ER- α 36 function in resistance of the breast cancer stem/progenitor cells to antiestrogens (months 17-24, from SOW).

We will focus our research on the PI3K/AKT signaling pathway to examine the possibility of down-regulation of ER- α 36 expression or inhibition of the PI3K/AKT pathway will sensitize antiestrogen resistant breast cancer stem/progenitor cells to antiestrogens.

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Appendix

Estrogen Receptor- α Variant, ER- α 36, is Involved in Tamoxifen Resistance and Estrogen Hypersensitivity

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Short Title: ER- α 36 in tamoxifen resistance and estrogen hypersensitivity

Key Words: ER- α 36, breast cancer, tamoxifen resistance, estrogen hypersensitivity.

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Abstract

Anti-estrogens such as tamoxifen provided a successful treatment for ER-positive breast cancer for the past two decades. However, most breast tumors are eventually resistant to tamoxifen therapy. The molecular mechanisms underlying tamoxifen resistance have not been well established. Recently, we reported that breast cancer patients with tumors expressing high levels of ER- α 36, a variant of ER- α , less benefited from tamoxifen therapy than those with low levels of ER- α 36 expression, suggesting that gained ER- α 36 expression is one of the underlying mechanisms of tamoxifen resistance. Here, we investigated the function and underlying mechanism of ER- α 36 in tamoxifen resistance. We found that tamoxifen induced ER- α 36 expression and tamoxifen resistant MCF7 cells expressed high levels of ER- α 36. In addition, MCF7 cells with forced expression levels of ER- α 36 and H3396 cells expressing high levels of endogenous ER- α 36 were insensitive to tamoxifen. Knockdown of ER- α 36 expression in tamoxifen resistant cells with the shRNA method restored tamoxifen sensitivity. We also found tamoxifen acted as a potent agonist by activating phosphorylation of the AKT kinase in ER- α 36 expressing cells. Finally, we found that cells with high levels of ER- α 36 expression were hypersensitive to estrogen; activating ERK phosphorylation at pM range. Our results thus demonstrated that elevated ER- α 36 expression is one of the mechanisms by which ER-positive breast cancer cells escape tamoxifen therapy and provided a rationale to develop novel therapeutic approaches for tamoxifen resistant patients by targeting ER- α 36.

Introduction:

Since mitogenic estrogen signaling plays a pivotal role in development and progression of ER-positive breast cancer, treatment with antiestrogens such as tamoxifen (TAM) provides a successful treatment option for ER-positive breast cancer patients in the past two decades. However, despite the significant anti-neoplastic activity of TAM, most breast tumors are eventually resistant to TAM therapy, which largely affects the efficacy of this treatment. Essentially, two forms of TAM resistance occur: *de novo* and acquired resistance (reviewed in 1, 2). Although absence of ER- α expression is the most common *de novo* resistance mechanism, about 50% ER-positive breast cancer patients with advanced disease do not respond to TAM treatment by the time of diagnosis (reviewed in 2). The exact mechanisms underlying the *de novo* TAM resistance in these ER-positive tumors are largely unknown. Several mechanisms have been postulated to be involved in the TAM resistance such as increased growth factor signaling, metabolism of TAM by CYP2D6 variants, altered expression of co-regulators, mutations of ER- α (reviewed in 3, 4). In addition, most initially responsive breast tumors gradually acquire TAM resistance by loss of TAM responsiveness, the acquired resistance. The mechanisms by which breast tumors lose their TAM responsiveness have not been well established. Breast tumors with acquired TAM resistance frequently retain levels and location of ER- α expression that would still classify them as ER-positive tumors (3). Therefore, a loss of ER- α expression is not a major mechanism driving acquired TAM resistance. Another acquired TAM resistance phenotype has been described in breast cancer xenografts that exhibit a switch from a TAM-inhibitory phenotype to a TAM-stimulated one (5,6). The agonist activity of TAM in this model may be due to the enhanced growth factor signaling that is often associated with acquired TAM resistance (reviewed in 7). However, the molecular mechanism underlying this type of acquired TAM resistance has not been well established.

During development of acquired antiestrogen resistance, breast cancer cells usually undergo adaptive changes in response to inhibitory effects of antiestrogens (8). Adaptive changes also occur in response to aromatase inhibitor therapy in post-menopausal patients or from oophorectomy in pre-menopausal patients (9, 10). Using a MCF7 breast cancer model system, Santen's group demonstrated that deprivation of estrogen for a prolong period of time confers these cells hypersensitive to low concentrations of estrogen (11). In these hypersensitive cells, 17- β -estradiol (E2) stimulates cell proliferation at pM range while the wild-type cells require nM range E2 to induce cell growth (11). However, the exact molecular events underlying the development of this "adaptive hypersensitivity" have not been elucidated although up-regulation and membrane localization of ER- α , activation of the non-genomic estrogen signaling, as well as induction of c-myc and c-myb have been proposed to be involved in this process (8, 12).

Previously, our laboratory identified and cloned a variant of ER- α , ER- α 36, which has a molecular weight of 36-kDa (13, 14). The transcript of ER- α 36 is initiated from a previously unidentified promoter in the first intron of the ER- α gene (15). This ER- α differs from the original 66 kDa ER- α (ER- α 66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains and partial ligand-binding domain (13). ER- α 36 is mainly expressed on the plasma membrane and mediates membrane-initiated estrogen signaling (14). We also found that the breast cancer patients with tumors expressing high levels of ER- α 36 less benefited from TAM therapy than those with low levels of ER- α 36 expression (16), suggesting that gained ER- α 36 expression is one of the underlying mechanisms of TAM resistance. Recently, we also reported that ER- α 36 is able to mediate agonist activity of TAM and ICI 182, 780 (17, 18) such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways.

Based on these observations, we hypothesized that ER- α 36 is involved in TAM resistance by

mediating agonist activity of tamoxifen. Using ER-positive breast cancer MCF7 cells that express high levels of recombinant ER- α 36 as a model system, we investigated the function of non-genomic TAM signaling by ER- α 36 in TAM resistance. Here, we present evidence to demonstrate that ER- α 36 plays an important role in development of TAM resistance presumably through activation of agonist activity of TAM, and estrogen hypersensitivity.

Materials and Methods:

Chemicals and Antibodies

17 β -estradiol (E2) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho44/42 ERK (Thr202/Tyr204) (197G2) mouse monoclonal antibody (mAb) and anti-p44/42 ERK (137F5) rabbit mAb were purchased from Cell Signaling Technology (Boston, MA). Antibodies of ER- α 66 and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-ER- α 36 antibody was generated and characterized as described before (14).

Cell culture and establishment of stable cell lines—MCF7 cells (ATCC) and its derivatives as well as H3396 cells (a kind gift from Dr. Leia Smith of Seattle Genetics) were maintained at 37°C in a 10% CO₂ atmosphere in IMEM without phenol red and 10% fetal calf serum. To establish stable cell lines with ER- α 36 expression knocked down, we constructed an ER- α 36 specific shRNA expression vector by cloning the DNA oligonucleotides 5'-GATGCCAATAGGTACTGAATTGATATCCGTTTCAGTACCTATTGGCAT-3' from the 3'UTR of ER- α 36 cDNA into the pRNAT-U6.1/Neo expression vector from GenScript Corp. Briefly, cells transfected with the empty expression vector and ER- α 36 shRNA expression vector were selected with 500 μ g/ml G418 for three weeks, and more than 20 individual clones from transfected cells were pooled, examined for ER- α 36 expression with Western blot analysis and retained for experiments. For ERK activation assays, cells were treated with vehicle (ethanol) and indicated concentrations of TAM.

To examine cell growth in the presence or absence of antiestrogens, cells maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped fetal calf serum (HyClone, Logan, UT) were treated with different concentrations of tamoxifen, 17 β -estradiol or ethanol vehicle as a control. The cells were seeded at 1 X 10⁴ cells per dish in 60mm dishes and the cell numbers were determined using the ADAM automatic cell counter (Digital Bio., Korea) after seven days. Five dishes were used for each treatment and experiments were repeated more than three times.

Western blot analysis-- For immunoblot analysis, cells washed with PBS were lysed with the lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 0.25 mM EDTA pH8.0, 0.1% SDS, 1% Triton X-100, 50 mM NaF) supplemented with protease and phosphatase inhibitors (Sigma). The protein amounts were measured using the DC protein assay kit (BIO-RAD Laboratories, Hercules, CA). The same amounts of the cell lysates were boiled for 5 minutes in loading buffer and separated on a SDS-PAGE gel. After electrophoresis, the proteins were transferred to a PVDF membrane. The membranes were probed with various primary antibodies, HRP-conjugated secondary antibodies, and visualized with enhanced chemiluminescence (ECL) detection reagents (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

Statistical analysis

Data were summarized as the mean \pm standard error (SE) using the GraphPad InStat software program. Tukey-Kramer Multiple Comparisons Test was also used, and the significance was accepted for $P < 0.05$.

Results:

Tamoxifen induce ER- α 36 expression in ER-positive breast cancer MCF7 cells.

Previously, our laboratory identified and cloned a 36 kDa variant of ER- α , ER- α 36 that functions differently from the 66 kDa full-length ER- α , ER- α 66 (11). Using a ER- α 36 specific antibody, we further found that ER- α 36 is highly expressed in established ER-negative breast cancer

cells while weakly expressed in ER-positive breast cancer cells such as MCF7 (11). In order to investigate ER- α 36 function in the activities mediated by antiestrogens, we first examined whether TAM influences ER- α 36 expression in MCF7 cells. The levels of ER- α 36 in MCF7 cells treated with 1 μ M of TAM for different time periods were examined with Western blot analysis. After treatment of TAM, the levels of ER- α 36 expression were dramatically increased in MCF7 cells (Figure 1), indicating that TAM is able to induce ER- α 36 expression in MCF7 cells.

TAM-resistant ER-positive breast cancer MCF7 cells express high levels of ER- α 36

To examine the involvement of ER- α 36 in development of TAM resistance, we cultured MCF7 cells in the presence of TAM (1 μ M) for six months and pooled all survived cells to establish a cell line MCF7/TAM. This cell line exhibited insensitivity to TAM treatment compared to the parental cells and TAM at 1 μ M even acted as an agonist in MCF7/TAM cells (Figure 2A). Western blot analysis revealed that ER- α 36 was expressed at a higher level in MCF7/TAM cells compared to the MCF7 parental cells, while ER- α 66 expression was without significant change (Figure 2B), suggesting that MCF7 cells gained ER- α 36 expression during development of acquired TAM resistance.

High levels of ER- α 36 expression confer TAM resistance

To confirm that gained expression of ER- α 36 is involved in development of TAM resistance, we sought to knockdown ER- α 36 expression in MCF7/TAM cells using the shRNA approach. We established a cell line that expressed knocked-down levels of ER- α 36 (MCF7/TAM/Si36) from MCF7/TAM cells as evidenced by Western blot analysis (Fig. 3A). MCF7/TAM cells with the knocked-down levels of ER- α 36 expression restored sensitivity to TAM, at a level similar to parental MCF7 cells (Fig. 3A). Our data thus suggested that elevated ER- α 36 expression is involved in development of acquired TAM resistance.

To further confirm high levels of ER- α 36 expression contributes to TAM resistance, we introduced recombinant ER- α 36 into MCF7 cells that express high levels of ER- α 66 but lower levels of ER- α 36 to establish a stable cell line, MCF7/ER36. Western blot analysis demonstrated that recombinant ER- α 36 was highly expressed in MCF7/ER36 cells compared to the control MCF7 cells transfected with the empty expression vector (Fig. 4A). When MCF7/ER36 cells were treated with different concentrations of tamoxifen, these cells are more resistant to growth inhibitory effects of TAM compared to the control MCF7 cells transfected with the empty expression vector (Fig. 3B), indicating that high levels of ER- α 36 expression is one of the underlying mechanism of tamoxifen resistance. We also found a breast cancer cell line H3396 that expressed high levels of endogenous ER- α 36 at a level comparable to MCF7/ER36 cells (Fig.4A). Like MCF7/ER36 cells, H3396 cells exhibited insensitivity to TAM treatment (Fig.4B).

Tamoxifen acts as a potent agonist in cells express high levels of ER- α 36.

Previously, we found that TAM elicited agonist activities such as activation of the MAPK/ERK and the PI3K/AKT pathways in ER- α 36 expressing endometrial cells (15,16). We sought to determine whether ER- α 36 mediates agonist activity of TAM in cells expressing high levels of ER- α 36. We first treated MCF7 cells with different concentrations of TAM and the levels of AKT phosphorylation was analyzed with Western blot analysis. In control MCF7 cells transfected with the empty expression vector (MCF7/Vector), we found that at lower concentrations (1-3 μ M), TAM induced the levels of AKT phosphorylation while at 4-5 μ M failed to do so. However, TAM failed to induce AKT activation in MCF7 cells with knocked-down levels of ER- α 36, indicating ER- α 36 mediates agonist activity of TAM. However, in MCF7/TAM, H3396 and MCF7/ER36 cells, TAM potently induced AKT activation even at 4-5 μ M compared to the control MCF7 cells (Fig. 5). To further confirm the role of ER- α 36 in the agonist function of TAM, we also used MCF/TAM and H3396 with knocked-down

levels of ER- α 36 expression. We found that TAM failed to act as a potent agonist in these cells (data not shown). Taken together, these results demonstrated that ER- α 36 mediates agonist activity of tamoxifen, which provides an explanation to the contribution of ER- α 36 in TAM resistance.

ER- α 36 expressing breast cancer cells exhibits estrogen hypersensitivity.

Previously, it was reported that cells deprived of estrogen for a long-term exhibited hypersensitivity to estrogens (7). We decided to examine whether ER- α 36 is involved in development of estrogen hypersensitivity. MCF7/TAM cells were treated with different concentrations of 17 β -estradiol (E2) for different time periods. We found that E2 stimulated stronger proliferation in these cells compared to the parental MCF7 cells (Fig. 5A). In addition, MCF7/TAM cells exhibited hypersensitivity to E2; at pM range, E2 strongly stimulated proliferation of MCF/TAM cells (Fig. 6A) while stimulated proliferation of the parental MCF7 cells at nM range. We also found that MCF7/ER36 cells that express high levels of recombinant ER- α 36 and H3396 cells with high levels of endogenous ER- α 36 also exhibited estrogen hypersensitivity (Fig. 6B), suggesting that ER- α 36 is involved in estrogen hypersensitivity.

We then examined E2-induced phosphorylation of the MAPK/ERK1/2, a typical non-genomic estrogen-signaling event, in different cells. Cells were treated with E2 at different concentrations, and Western blot analysis with a phospho-specific ERK1/2 antibody was performed. Figure 6C shows that E2 elicited ERK phosphorylation in MCF/TAM cells in a dos-dependent manner starting at a extreme low concentration, 1×10^{-14} M/L while in the parental MCF7 cells, ERK activation requires E2 at 1×10^{-14} M/L (Fig. 6B). A similar hypersensitivity was also observed in MCF7/ER36 and H3396 cells; E2 induced ERK phosphorylation at 1×10^{-14} M/L.

Discussion:

Tamoxifen therapy is the most effective treatment for advanced ER-positive breast cancer, but

its effectiveness is limited by high rate of *de novo* resistance and resistance acquired during treatment. Many researches were conducted to understand the molecular pathways responsible for the *de novo* and acquired tamoxifen resistance and have revealed that multiple signaling molecules and pathways are implicated in tamoxifen resistance. All these pathways often bypass the requirement of estrogen signaling pathway for growth of ER-positive breast cancer cells. Previously, we reported that the breast cancer patients with tumors expressing high levels of endogenous ER- α 36 less benefited from tamoxifen therapy than those with low levels of ER- α 36 expression (16), suggesting elevated expression of ER- α 36 may be a novel mechanism underlying both *de novo* and acquired tamoxifen resistance.

Here, we showed that tamoxifen treatment induced ER- α 36 expression and tamoxifen resistant MCF7/TAM cells selected by long-term cultivation in the presence of tamoxifen expressed elevated levels of endogenous ER- α 36. We also showed that MCF7 cells with forced expression levels of ER- α 36 and H3396 cells that express high levels of endogenous ER- α 36 were insensitive to tamoxifen. Knockdown of ER- α 36 expression level, however, was able to restore tamoxifen sensitivity in MCF/TAM and H3396 cells, indicating that increased ER- α 36 expression is one of the molecular mechanisms by which ER-positive breast cancer develops tamoxifen resistance.

Previously, we found that antiestrogens TAM and ICI 182, 780 failed to block ER- α 36 mediated non-genomic estrogen signaling (14). Here we showed that TAM exhibited a biphasic activation of the AKT kinase in tamoxifen sensitive MCF7 cells; increasing AKT phosphorylation at low concentrations and failed to do so at higher concentrations. However, in cells expressing high levels of ER- α 36, TAM acted as a potent agonist to strongly activate the AKT kinase, consistent with our recent report that ER- α 36 mediates agonist activities of both TAM and ICI 182, 780 in cells that express high levels of endogenous ER- α 36 (18). Our results thus indicate that ER- α 36 is involved in TAM resistance

presumably through mediating agonist activity of TAM.

Previously, another acquired TAM resistance phenotype has been described in human breast cancer xenografts that exhibit a switch from a TAM-inhibitory phenotype to a TAM-stimulated one. Some breast cancers may be initially growth inhibited by TAM, and later become dependent on TAM for proliferation (19-21). These xenografts also retain the ability to be stimulated by estrogens (19-21). In the current study, we found that 1 μ M of TAM stimulated proliferation of MCF7/TAM cells while knockdown of ER- α 36 expression in these cells diminished TAM-stimulation. In addition, these TAM resistant cells retained estrogen responsiveness, and even showed estrogen hypersensitivity. Our results thus suggested that elevated ER- α 36 expression is involved in this type of TAM resistance. It also worth noting that the TAM at 1 μ M failed to stimulate proliferation of MCF7/36 cells that express high levels of recombinant ER- α 36, suggesting that gained ER- α 36 expression alone is unable to render TAM stimulatory phenotype.

Previously, it has been reported that physiological concentrations of E2 exhibits antitumor activity in a TAM-stimulated MCF7 cell model that generated by serial transplantation of TAM resistant tumors the continuous presence TAM (22). Based on the laboratory studies, recently, it was proposed that physiological concentrations of estrogen could be used as an therapeutic approach for these TAM resistant patients (23, 24). However, the molecular mechanisms underlying this paradoxical phenomenon have not been well elucidated. It is known that estrogen stimulates growth of ER-positive breast cancer cells in a biphasic growth curve; stimulating cell proliferation at low concentrations while failing to stimulate or even inhibiting cell growth at higher concentrations. Our evidence presented here that elevated ER- α 36 rendered cells hypersensitive to E2; shifting the biphasic growth curve to the left. Thus, in cells expressing high levels of ER- α 36, physiological concentrations of E2 may fail to stimulate proliferation or even inhibit proliferation of ER-positive breast cancer cells. Our data thus provided a

molecular explanation to the paradoxical phenomenon that some TAM resistant tumors are simulated by TAM but inhibited by estrogen.

Previously, it was reported that long-term estrogen deprivation with hormonal therapy resulted in “adaptive” changes in breast cancer cells; making these cells hypersensitive to estrogen (8, 11). Recently, we reported that the expression levels of ER- α 36 is dramatically increased in normal osteoblasts cells from menopausal women (25), suggesting the expression levels of ER- α 36 is elevated in response to low concentrations of estrogen in menopausal women. Our current data showed that E2 induced ERK phosphorylation and stimulated proliferation at pM range in cells with high levels of ER- α 36 expression while at nM range in cells with low levels of ER- α 36. Thus, our results indicated that gained expression of ER- α 36 is one of the “adaptive” changes in breast cancer cells after a long-term estrogen deprivation.

In summary, here we provided evidence to demonstrate that ER- α 36 is a novel and important player in normal and abnormal estrogen signaling, and ER- α 36 is involved in many physiological and pathological processes regulated by estrogen signaling. Our findings that elevated ER- α 36 expression is one of the mechanisms by which ER-positive breast cancer cells escape the hormonal therapy based on estrogen deprivation provided a rational to develop novel therapeutic approaches for antiestrogen resistant patients by targeting ER- α 36.

Figure legends

Fig.1. Tamoxifen induces ER- α 36 expression. Western blot analysis of the expression levels of ER- α 36 and 66 in ER-positive breast cancer MCF7 cells treated with 1 μ M of tamoxifen.

Fig.2. Tamoxifen resistant ER-positive breast cancer MCF7 cells express high levels of endogenous ER- α 36. A. ER-positive breast cancer MCF7 cells and tamoxifen resistant MCF7 cells (MCF7/TAM) cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and survived cells were counted. The columns represent the means of three experiments; bars, SE. B. Western blot analysis of the expression levels of ER- α 36 and 66 in MCF7 and MCF7/TAM cells.

Fig.3. ER- α 36 is involved in tamoxifen resistance. A. Western blot analysis of the expression levels of ER- α 36 and 66 in MCF7 cells, MCF7/TAM cells transfected with the empty expression vector (MCF/TAM/Vector) and MCF7/TAM cells transfected with an ER- α 36 specific shRNA expression vector (MCF7/TAM/Si36). B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and the numbers of survived cells were counted. The columns represent the means of three experiments; bars, SE. *, $P < 0.05$ for MCF/TAM/Vector cells treated with vehicle vs cells treated with 1 μ M of tamoxifen.

Fig.4. ER-positive breast cancer cells with elevated levels of ER- α 36 expression are resistant to tamoxifen. A. Western blot analysis of the lysates from ER-positive breast cancer MCF7 cells, MCF7 cells with forced expression of ER- α 36 (MCF7/ER36) and H3396 cells with high levels of endogenous ER- α 36. B. Cells were treated with indicated concentrations of tamoxifen (TAM) for seven days and survived cells were counted. The columns represent the means of three experiments; bars, SE.

Fig.5. ER- α 36 mediates tamoxifen-induced phosphorylation of the AKT kinase in ER-positive breast cancer MCF7 cells. A. Western blot analysis of ER- α 36 and 66 expression levels in MCF7 cells transfected with an empty expression vector (MCF7/Vector) and MCF7 cells transfected with the ER- α 36 specific shRNA expression vector (MCF7/Si36). B. Western blot analysis of phosphorylation levels of AKT in different cells treated with indicated concentrations of tamoxifen (TAM) using phospho-specific or non-specific AKT antibodies.

Fig.6. ER- α 36 is involved in estrogen hypersensitivity. A. ER-positive breast cancer MCF7 cells and MCF7/TAM cells were treated with indicated concentrations of 17 β -estradiol (E2) for seven days and cell number was counted. Each point represents the means of three experiments; bars, SE. B. MCF7 cells transfected with the empty expression vector (MCF7/Vector) and ER- α 36 shRNA expression vector (MCF7/ER36) as well as H3396 cells were treated with indicated concentrations of E2 for seven days and cell number was counted. Each point represents the means of three experiments; bars, SE. C. Estrogen-induced ERK activation in different cells. Western blot analysis of the lysates from different cells treated with different concentrations of E2 with phospho-specific or non-specific ERK1/2 antibodies.

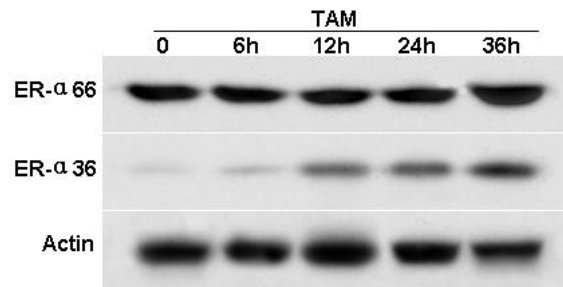
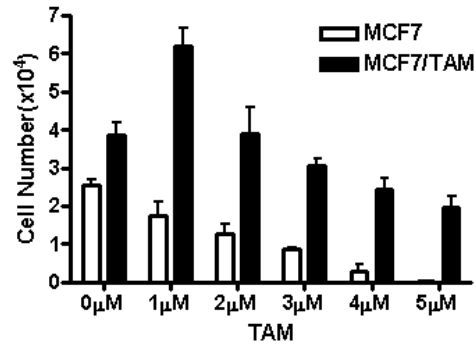


Fig. 1 Zhang et.al.

A.



B.

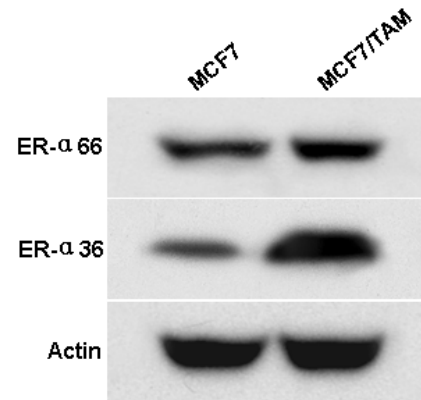
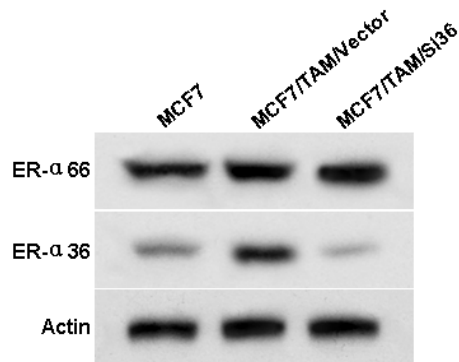


Fig. 2 Zhang et.al.

A.



B.

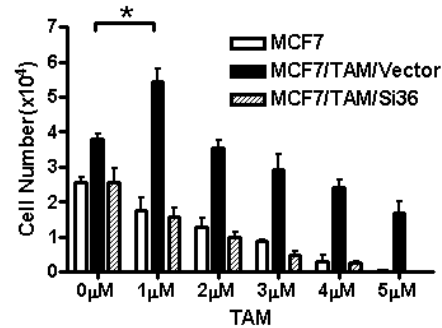
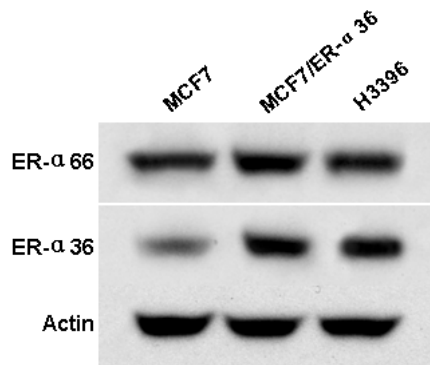


Fig.3 Zhang et.al.

A.



B.

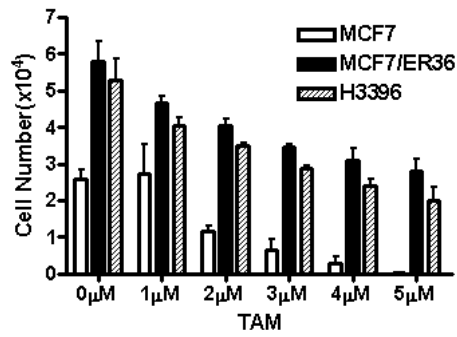
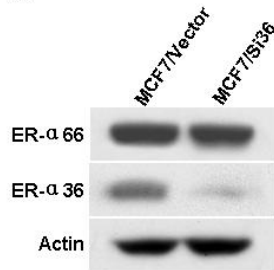
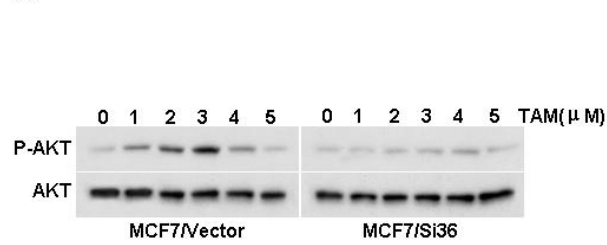


Fig. 4 Zhang et.al.

A.



B.



C.

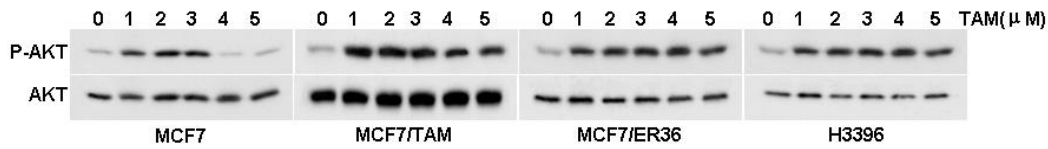


Fig. 5 Zhang et.al.

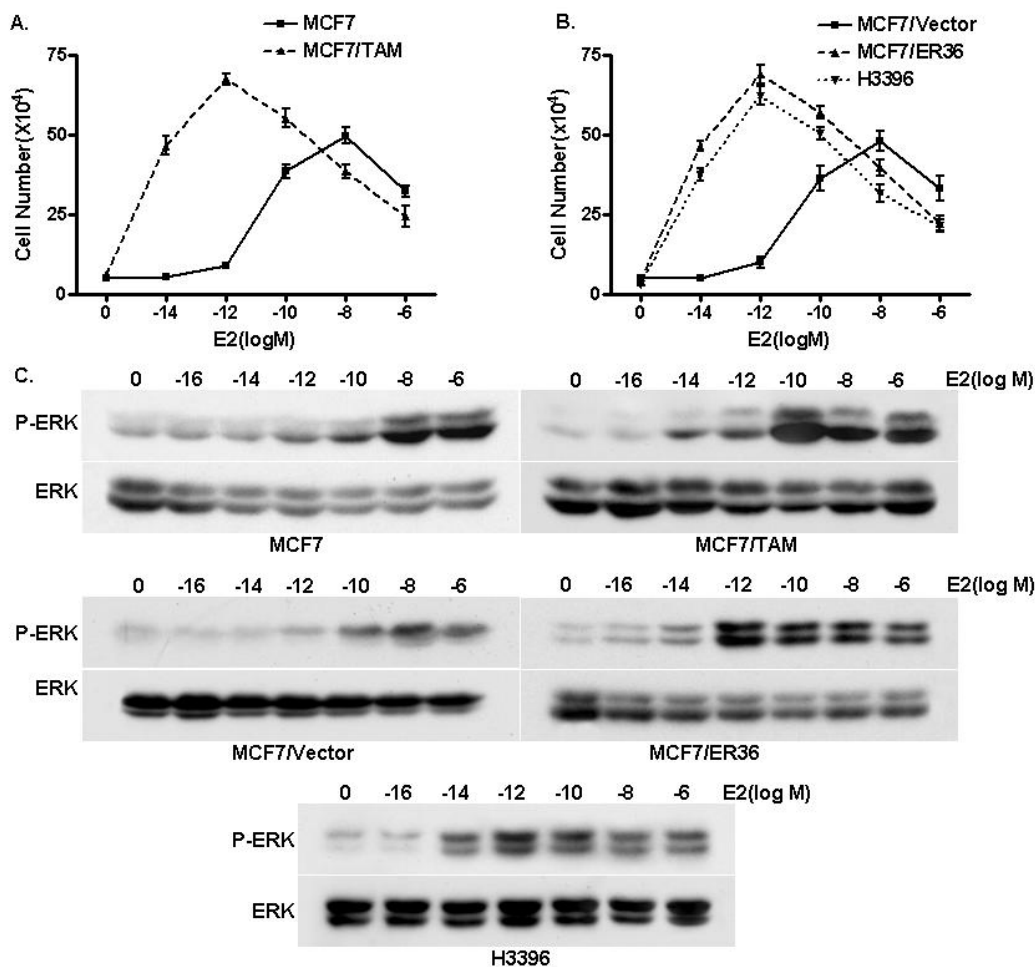


Fig. 6 Zhang et.al.

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